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Bovine Papillomavirus Type 1 E1 and Simian Virus 40 Large T Antigen Share Regions of Sequence Similarity Required for Multiple Functions

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The full-length product of the bovine papillomavirus type 1 (BPV-1) E1 translational open reading frame is required for viral DNA replication in vivo and in vitro. E1 is a multifunctional protein whose properties include ATP binding, acting as an ATPase-dependent DNA helicase, DNA binding to the BPV-1 origin of viral DNA replication, and association with the E2 transcriptional transactivator, E2TA, a second viral protein involved in DNA replication. All of these properties are thought to be important for E1's role in replicating the viral genome. In addition BPV-1 E1 can inhibit activation of the viral P_{89} promoter by the BPV-1 E2TA. E1 has amino acid homology with eight regions of SV40 large tumor antigen (T-ag), a DNA helicase that is essential for the replication of the SV40 DNA genome. These eight regions of similarity lie within the domain of T-ag that confers DNA helicase activity. We created a series of missense mutations in BPV-1 E1 at codons 295, 344-345, 446, 464, 466, 497-498, 523, and 542, which encode amino acids of identity in seven of the eight regions of similarity between E1 and T-ag, and at codon 370. The activities of these mutant E1 genes were compared to wild-type E1 in multiple assays that measured DNA replication, inhibition of E2TA-dependent transcription, DNA binding, ATP binding, and protein expression. Based upon these analyses, the following conclusions were made: (i) at least five of the eight regions in E1 that are similar to regions in T-ag are functionally important in viral DNA replication; (ii) specific E1 missense mutants, themselves defective for supporting DNA replication, could act in *trans* to suppress the replication function of wild-type E1; (iii) certain regions of similarity with T-ag that are important for E1's ability to support DNA replication are not necessary for its capacity to inhibit E2TA-dependent transcription; and (iv) efficient DNA binding by E1 is not essential for E1 to inhibit E2TA-dependent transcription.

Papillomaviruses are small DNA tumor viruses that cause benign epithelial proliferation which in certain cases proceeds to malignancy after long latency periods. Bovine papillomavirus type 1 (BPV-1) is associated with fibropapillomas in the skin of cattle, where, in addition to proliferation of the epithelial cells, there is also a proliferation of the dermal fibroblasts (28). BPV-1 can transform mouse C127 cells in culture, and in these cells, the viral early genes are expressed and the viral DNA is replicated as a plasmid at 50 to 100 copies per cell without the production of progeny virus (16). The infected state found in virally transformed C127 cells is thought to reflect the early stages of papillomavirus infection found within the undifferentiated epithelium of BPV-1-induced warts.

By using virally transformed mouse C127 cells as a tissue culture model for studying a portion of the papillomavirus life cycle, viral genes required for viral DNA replication have been identified. The BPV-1 E1 protein, a nuclear phosphoprotein, and the BPV-1 E2 transcriptional transactivator (E2TA) are the only viral proteins necessary for viral DNA replication (42, 46). E1 has been shown to associate with E2TA (2, 21, 26), and this protein association may be important for viral DNA replication (34). Sequences required in *cis* for viral DNA replication (BPV-1 nucleotides [nt] 7914 to 27), referred to as the origin of viral DNA replication, contain binding sites for both the E1 and E2TA proteins (13, 42). BPV-1 E1 has weak affinity for its DNA binding site in the origin; E2TA, a transcriptional transactivator with a higher DNA binding affinity, is thought to target E1 to the origin of replication through its association with E1 (26, 31, 34). Recently it has been suggested that E2TA might facilitate the stepwise assembly of E1 into a multimeric complex akin to the hexameric form of simian virus 40 (SV40) large tumor antigen (T-ag), which assembles at the SV40 origin (5, 7, 9, 22, 24, 35). Like T-ag, E1 is able to bind ATP (23, 40), act as an ATPase-dependent DNA helicase (36, 47), and bind DNA polymerase alpha (4), all properties thought to be important for E1's role in viral DNA replication.

In addition to being defective for viral DNA replication, BPV-1 genomes defective for E1 expression are altered in their transcriptional regulation. Compared to wild-type BPV-1, E1-defective mutants display a three- to ninefold increase in the amount of viral-specific RNA per viral genome in cells, and this increase is associated with a selective increase in the steady-state levels of RNAs arising from only two viral promoters, P_{89} and P_{2443} (15, 33). Cells harboring E1 mutants also display a fivefold increase in E2TA-dependent transcriptional transactivation compared to cells harboring wild-type BPV-1 (15). Sandler et al. (30) demonstrated that E1 can repress E2TA-transactivated transcription from the viral promoter P_{89} in bovine embryo fibroblasts (BEF). They determined that transcriptional repression by E1 correlated with the capacity for the E1-E2TA complex to bind the replication origin, which is just upstream of the P_{89} promoter. However, a mutant of E2TA unable to support viral DNA replication was as effi-

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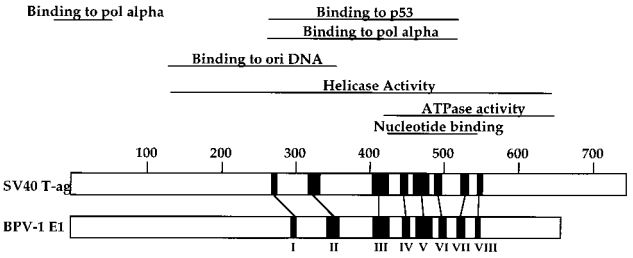
A.



B.

Region	Amino Acid Residue Numbers		Amino Acid Sequence	Mutation Made
I	T-ag	269-270	*sW*	
	E1	294-295	*EW*	W295R
II	T-ag	323-333	*NAaIFADsknQ*	
	E1	344-354	*NArAFLaTnsQ*	N344H/A345E
IV	T-ag	444-453	*LLeLcGGKAL*	
	E1	446-455	*LIhfLGGsVL*	L446K
V	T-ag	459-475	*FeLqvAIDqfLVVfEDV*	
	E1	464-480	*FwLasLADtrAALvDDA*	F464C and L466H
VI	T-ag	497-513	*LRDyLDGsvkVnLEKKH*	
	E1	492-507	*LRNaLDGypVsIDRKH*	D497A/G498A
VII	T-ag	522-529	*PPaIVTmN*	
	E1	516-523	*PPlLVTsN*	N523I
VIII	T-ag	547-549	*FRp*	
	E1	542-544	*FRf*	F542V

FIG. 1. Amino acid similarities and positions of E1 mutations. (A) The black bands indicate the relative positions in SV40 T-ag and BPV-1 E1 proteins of the eight domains of amino acid similarity. Above the structure diagram are indicated the functional domains that have been mapped in SV40 T-ag, which overlap the eight domains of amino acid similarity. (B) Amino acid compositions of the seven domains of amino acid similarity in which mutations were made in E1. Capital letters indicate amino acid residues that are conserved among E1 species or among T-ag species of SV40 and polyoma viruses. Italicized capital letters indicate amino acid residues conserved among both E1 species and T-ag species of SV40 and polyoma viruses. Also indicated are the amino acids that were targeted for mutagenesis in BPV-1 E1. pol alpha, DNA polymerase alpha.

ciently inhibited by E1 in its activation of P_{89} transcription as was wild-type E2TA. This observation indicated that the inhibition of transcription by E1 is not dependent upon replication of the template (30). Le Moal et al. (17) determined that the E1 binding site within the origin was not required for E1's ability to inhibit E2TA transactivation of the P_{89} promoter and that E1 could modulate E2TA activity on a heterologous promoter when E1 was added at low doses.

Clertant and Seif (6) reported that E1 and SV40 T-ag have amino acid similarity in eight distinct regions (Fig. 1A). The portion of T-ag encompassing these eight regions encodes domains necessary for ATP binding, ATPase-dependent DNA helicase activity, and DNA polymerase alpha binding, all properties important for T-ag's role in SV40 DNA replication (8). In this study, amino acid substitutions were made in BPV-1 E1 at conserved residues within seven of the eight regions of homology between SV40 T-ag and BPV-1 E1 and their effects on biological properties of E1 were analyzed. We predicted that these conserved amino acid residues would be important for E1's role in replication because, when corresponding mutations were made in T-ag, its ability to support viral DNA replication was disrupted (1, 20). Amino acid substitutions made in E1 led to defects in E1's ability to support viral DNA replication, indicating that the similarity between T-ag and E1 is biologically significant. Two of the E1 missense mutants could act in *trans* to inhibit replication by wild-type E1. Five E1 mutants that were defective for DNA replication were competent to inhibit E2TA-mediated transcription, demonstrating that the replication activities of E1 are separable from its transcriptional regulatory activities. Of these five mutants, one was decreased in its DNA binding activity by more than 100-fold. The significance of these findings in the context of our understanding of E1's role in transcription and replication is discussed.

MATERIALS AND METHODS

Generation of E1 mutants. To generate the amino acid substitutions in the E1 open reading frame, oligonucleotide-directed site-specific mutagenesis was performed as previously described (14). Briefly, fragments of BPV-1 containing portions of the E1 open reading frame, from BPV-1 nt 945 to 2113 and 2113 to 3456, were cloned into M13mp19 and M13mp18, respectively. The recombinant M13 phages were used to infect cultures of *Escherichia coli* CJ236 (*dut ung*) to generate single-stranded M13 genomic DNA templates containing uridine residues in place of thymidine. Oligonucleotides containing base pair mismatches that would cause specific missense mutations in E1 (listed in Fig. 1) were annealed to the single-stranded M13 template that contained complementary E1 sequences, and the negative strand was synthesized by the addition of T4 DNA polymerase and deoxynucleoside triphosphates. The DNA products were transfected into *E. coli* JM109 (dut^+ ung^+), and viral genomes from individual plaques were sequenced to determine if the genome contained the appropriate mutation. Double-stranded DNA replicative forms of the recombinant M13 viruses containing the mutations were digested with either *Sma*I and *Eco*RI or *Eco*RI and *Avr*II to release BPV-1 fragments, which were then cloned into pMH142-6, a bacterial plasmid that contains the full-length BPV-1 DNA genome (32). The mutations were also cloned into pCGEag1235$^-$, an E1 expression vector that contains a silent point mutation at BPV-1 nt 1235 that disrupts a 5′ splice signal within the E1 gene (42), by replacing the *Sma*I-to-*Bst*EII or *Sph*I-to-*Sph*I E1-specific DNA fragments in pCGEag1235$^-$ with equivalent fragments from the pMH142-6 derivatives containing the point mutations. The mutations were also cloned into the pGEX-E1 (4) vector by replacing the *Bsp*EI-to-*Avr*II E1-specific DNA fragment in pGST-E1 with the corresponding DNA fragment from the derivatives of pMH142-6 that contain the individual E1 point mutations. In all cases, plasmids were sequenced to verify that they contained the appropriate E1 point mutations.

Production of polyclonal antibody. His-tagged BPV-1 E1 protein was produced in *E. coli* harboring the plasmid pET/E1/His, which contains the full-length E1 gene (nt 849 to 2679) placed in frame downstream of the poly-His linker in pET-16B. Fifty micrograms of an insoluble protein fraction extracted from these bacteria, and containing predominantly the His-tagged BPV-1 E1 protein, was mixed with complete Freund's adjuvant and injected intradermally into a rabbit. Five boosts (equivalent amounts of E1 protein in incomplete Freund's adjuvant) were given to the rabbit at 1-month intervals. Two weeks after each injection serum was collected. Serum was analyzed for specificity for E1 by Western blot and immunoprecipitation (IP) analyses.

Detection of E1 protein in COS cells. Five micrograms of pCGEag1235$^-$ (wild type or mutant) was transfected into COS cells (6-cm-diameter dish) by the DEAE-dextran method (10). Two days posttransfection, the medium from the cells was removed and they were washed two times in phosphate-buffered saline. Cells were incubated in RIPA (50 mM Tris [pH 7.4], 150 mM NaCl, 10 mM EDTA, 1% Triton X-100, and 1% deoxycholic acid) on ice in the cold room (4°C) for 30 min. The cell lysate was scraped into microcentrifuge tubes, cellular DNA in the lysate was sheared by passing the lysate repeatedly through a 27-gauge needle, and then insoluble material was removed by centrifugation in an Eppendorf Microfuge (maximum speed) for 5 min at 4°C. The protein content of the supernatant was determined by Bradford (Bio-Rad) assay. Approximately 3 mg of total soluble protein was incubated at room temperature with 2 μl of the above-described E1-specific rabbit polyclonal antiserum in a total volume of 200 μl of RIPA for 1 h. Fifty microliters of 100 mg of protein A-Sepharose (Pharmacia)/ml was added to each sample, and incubation was continued for an additional hour with rocking at 4°C. After brief centrifugation in an Eppendorf Microfuge (maximum speed), the precipitates were washed three times in 400 μl of ice-cold RIPA. Sodium dodecyl sulfate (SDS) loading dye was added to the well-drained pellet, and the sample was boiled for 5 min prior to being loaded onto an SDS–8% polyacrylamide gel. Proteins were electrotransferred from the gel to polyvinylidene difluoride membrane (Millipore) at 100 V for 2 h in transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol). The blots were blocked overnight in phosphate-buffered saline containing 0.1% Tween 20 and 5% powdered milk and then incubated with primary antibody (anti-E1-specific rabbit sera diluted 1:1,000) in blocking solution for 1 h at room temperature with shaking. After brief washing the blot was incubated with secondary antibody (goat anti-rabbit antibody coupled to horseradish peroxidase, 1:10,000 dilution)

in phosphate-buffered saline containing 0.1% Tween 20 for 30 min at room temperature with shaking. Following multiple washes with phosphate-buffered saline containing 0.1% Tween 20, antibody staining was detected by enhanced chemiluminescence (ECL) according to the manufacturer's protocol (ECL Western detection kit; Amersham). Quantitation of E1 protein on Western blots was done by scanning the Western blot with a soft laser densitometer (Zenith).

Transient replication. For transient replication assays in which the replication of the full-length viral plasmid pMH142-6 was analyzed, 15 μg of pMH142-6 containing wild-type or mutant E1 was transfected into BEF in 100-cm-diameter dishes by calcium phosphate transfection (12). BEF cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Low-molecular-weight DNA was isolated at 4, 5, and 6 days posttransfection according to the modified Hirt DNA protocol described by Gopalakrishnan and Khan (11). To distinguish BPV-1 DNA replicated in the mammalian cells from input, bacterially synthesized DNA, the Hirt DNA was digested with *Dpn*I. The *Dpn*I-digested Hirt DNA was digested with *Hin*dIII to linearize the viral replicon, separated on a 0.8% agarose gel, and then analyzed by Southern blotting. The *Bam*HI fragment of pMH142-6 containing the entire BPV-1 genomic DNA was labeled with [α-^{32}P]dCTP by using a random primer labeling kit (Rediprime; Amersham). Replicated DNA was quantified with a Molecular Dynamics PhosphorImager.

In other transient replication assays, wild-type or mutant E1 was supplied in *trans* from pCGEag1235$^-$ or derivatives thereof and E2TA was supplied from pCGE2 (42). Replication was scored by using the plasmid pRLH89.7, which has BPV-1 nt 6958 to 93 (containing sequences required in *cis* for BPV-1 DNA replication, i.e., the minimal origin, *ori*) inserted into the backbone of pGL$_2$ basic vector (Promega). Eight micrograms of the pRLH89.7, 3 or 12 μg of pCGEag1235$^-$ (wild type or mutant), and 5 μg of pCGE2 were transfected into 1×10^7 to 2×10^7 C33A cells by electroporation at 180 V and 960 μF. C33A cells were grown in Dulbecco's modified Eagle medium supplemented with 10% bovine calf serum. Hirt DNA was isolated at 3 days posttransfection and digested with *Dpn*I and, to linearize the DNA, *Bgl*II. The digested Hirt DNA was analyzed as indicated above by Southern analysis. For the transient replication assays in which dominant-negative activities of mutant E1 genes were scored, C33A cells were transfected with the following amounts of DNA: 8 μg of pRLH89.7; 5 μg of pCGE2; 3 μg of wild-type E1 expression plasmid (pCGEag1235$^-$); either 0, 3, 6, 12, or 24 μg of E1 mutant expression plasmid (derivatives of pCGEag1235$^-$); and a sufficient amount of pCGEag*Sma* translation termination linker (TTL) (42), which does not express functional E1 or E2 proteins, to bring the total amount of pCG derivative plasmid DNA up to a constant of 32 μg of DNA. All other steps were performed as indicated above.

Repression of E2TA-mediated transcription. Assays were done as previously described (30). Briefly, 5 μg of pRLH89.7, varying amounts of pCGEag1235$^-$ (wild type or mutant), and 200 ng of pC59, which expresses E2TA (48), were transfected into BEF cells (80% confluent 6-cm-diameter dish) by the calcium phosphate precipitation method. The total amount of the pCG derivative DNA was adjusted to 5 μg with pCGEag*Sma* TTL. Cells were harvested 2 days posttransfection, and a luciferase assay (Promega) was performed according to the manufacturer's directions. Total cellular protein was quantitated by a Bradford assay (Bio-Rad). Equal amounts of total cellular protein were used in each luciferase assay.

DNA binding. McKay assays were performed as previously described (45) with a few modifications. Glutathione *S*-transferase (GST)-E1 fusion proteins (wild type or mutant) were expressed in *E. coli* HB101 and purified according to published methods (4). Two hundred fifty nanograms of the GST-E1 fusion proteins (wild type or mutant) was bound to glutathione-Sepharose 4B beads (Pharmacia) at 4°C for 30 min. Two sets of GST-E1 fusion proteins were bound to glutathione-Sepharose 4B beads. The beads were washed three times in 300 μl of buffer A (20 mM Tris-HCl [pH 8.0], 300 mM NaCl, 50 mM $MgSO_4$, 5 mM dithiothreitol, 1% Triton X-100, and 1× protease inhibitor cocktail [50× protease inhibitor cocktail is 2.5 mM tosyl-lysine chloromethyl ketone, 50 mM amino caproic acid, 50 mM benzamidine, 0.1 mM leupeptin, 0.1 mM pepstatin, 7% aprotinin, and 5% trypsin-chymotrypsin inhibitor in a buffer of 1 mM EDTA {pH 8} and 5 mM EGTA]). The plasmid pMH142-6 was digested with *Ava*II, end labeled with [α-^{32}P]dCTP, phenol extracted, and run through a G50 Sephadex spin column to remove unincorporated radionucleotides. Fifty nanograms of labeled BPV-1 DNA was added to one set of bound protein along with 25 μl of McKay binding buffer (TNE [10 mM Tris {pH 8.0}, 0.1 M NaCl, 1.0 mM EDTA {pH 8.0}] containing 150 mM NaCl and 1,500 ng of unlabeled sheared salmon sperm DNA) and incubated at room temperature for 30 min. To the second set of GST-E1 fusion proteins only McKay binding buffer was added. The protein and DNA mixture was washed with 1 ml of McKay wash buffer (TNE containing 200 mM NaCl, 0.25% Nonidet P-40, and 5 μg of sheared salmon sperm DNA) at room temperature three times. The second set of proteins was washed with McKay wash buffer, SDS loading dye was added, and samples were boiled and run in SDS–8% polyacrylamide gel electrophoresis (PAGE). The gel was stained with Coomassie blue to quantitate the amount of GST-E1 protein used in the DNA binding reactions. The protein-DNA complex was disrupted by incubating with 1% SDS at 37°C for 15 min and then phenol extracting the DNA. Loading dye (0.25% bromophenol blue, 0.25% xylene cyanole, 50% glycerol) was added to the DNA, and the DNA was resolved on an 8% polyacrylamide gel. The amount of nonspecific and specific DNA bound by each GST-E1 fusion was quantitated with a Molecular Dynamics PhosphorImager.

ATP binding. [α-^{32}P]ATP was incubated in 4 mM HCl–4 mM $NaIO_4$ for 30 min in the dark to generate an oxidative form, 2′,3′-dialdehyde ATP (ox-ATP), of ATP as previously described (40). The reaction was stopped by adding 0.25 volume of 50% glycerol for an additional 20 min to reduce any unreactive periodate. Two hundred fifty nanograms of GST E1 fusions was bound to glutathione-Sepharose 4B (Pharmacia) beads at 4°C for 30 min and then washed with 300 μl of buffer A three times. Two microliters of the ox-ATP was added to the bound protein along with 100 μl of ATP binding buffer (0.5 M KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM EGTA) and allowed to incubate on ice for 30 min. Then 0.02 volume of 0.5 M $NaCNBH_4$ was added to each reaction mixture to cross-link ox-ATP to protein, and the mixture was allowed to incubate overnight on ice. The next day the reactions were spun down and the supernatant was removed. The beads were washed two times with 300 μl of buffer A each time. SDS loading dye was added to the beads, and the protein was boiled and loaded onto an SDS–8% polyacrylamide gel. Before drying down the gel, it was stained with Coomassie blue to quantitate the amount of GST fusion proteins loaded in each lane. The amount of ox-ATP bound by each GST fusion was determined with a Molecular Dynamics PhosphorImager.

RESULTS

Generation of E1 mutants. Eight regions of amino acid similarity exist between SV40 T-ag and BPV-1 E1 (6). Nonconservative amino acid substitutions were made in seven of the eight regions within the BPV-1 E1 gene (Fig. 1B). In addition, a missense mutation was made at amino acid residue 370, owing to the fact that a similar mutation made at a conserved residue in T-ag (not originally identified by Clertant and Seif [6]) led to a defect in SV40 DNA replication (8). All of the amino acid substitutions were made at residues with identity among the E1 proteins of all sequenced papillomaviruses and with identity in SV40 T-ag. The mutations were made by oligonucleotide-directed, site-specific mutagenesis (14) and were confirmed by sequence analysis.

Expression levels of E1 proteins. To determine if the nonconservative amino acid substitutions in our E1 mutants disrupted the accumulation of E1 proteins in tissue culture cells, the steady-state level of each mutant E1 protein in cells was determined. Wild-type or mutant E1 genes that had been cloned into the vector pCGEag1235$^-$ were introduced into COS cells by DEAE-dextran transfection. The pCGEag1235$^-$ vector contains the SV40 origin of replication, allowing for its amplification in COS cells which express SV40 T-ag. This gene amplification was crucial to cause the expression of sufficient levels of E1 protein to allow detection. To determine whether equivalent levels of amplification occurred in each transfection, we isolated Hirt DNA from COS cells transfected with the pCGEag1235$^-$ vectors and analyzed it by Southern analysis following digestion with *Dpn*I. All vectors amplified to similar levels (data not shown). Protein lysates were extracted at the same time point, 2 days posttransfection, and analyzed by E1-specific IP combined with E1-specific Western analysis (Fig. 2). E1-specific signals on the Western blot were detected by ECL. Quantitation of ECL signals for each E1 protein was performed with a laser densitometer (Table 1). The mutants L446K, L466H, and N523I had steady-state levels of E1 protein below the limit of detection and were present at levels less than 10% that of wild-type E1 (Table 1). However, the mutant E1 protein L446K could be detected when twice as much extract as shown in Fig. 2 was immunoprecipitated (data not shown). Quantitation of the ECL signals in Fig. 2 indicated that two additional mutant proteins (D497A/G498A and F542V) accumulated to levels of 10% that of wild-type E1 protein (Table 1). The significance of these reduced levels of accumulation is considered in the discussion below.

Transient replication. To determine the effects of the amino acid substitutions on the ability of E1 to support viral DNA replication, transient replication assays were carried out. The

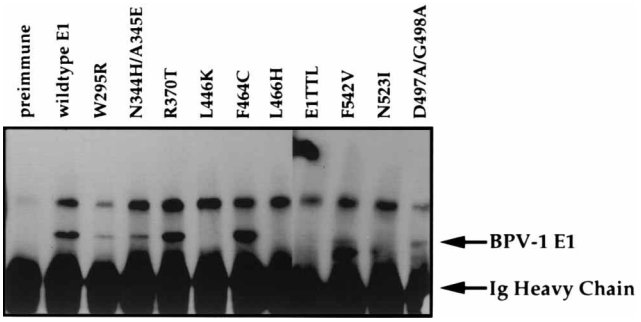


FIG. 2. Immunodetection of BPV-1 E1 proteins. COS cells were transfected with pCGEag1235$^-$ vectors expressing wild-type E1, amino acid substitution E1 mutants, or a mutant of E1 that contains a TTL at nt 945 (E1TTL). Equal amounts of total cell extracts were immunoprecipitated with E1-specific rabbit polyclonal sera, resolved by SDS-PAGE, and subjected to E1-specific immunoblotting. Arrows indicate the positions of migration of the E1 proteins and the immunoglobulin (Ig) heavy chain peptide brought down in the IP and detected by the secondary anti-rabbit antibody. Quantitation of wild-type or mutant E1 protein abundance from this and like experiments is provided in Table 1 (in vivo protein). Quantitation was based on scanning the Western blot with a soft laser densitometer (Zenith).

E1 genes containing the amino acid substitutions were cloned into the full-length BPV-1 genome in the context of the plasmid pMH142-6 and introduced into BEF by calcium phosphate transfection. Hirt DNA was isolated at 4, 5, and 6 days posttransfection, digested with *Hin*dIII and *Dpn*I, and analyzed by Southern hybridization with a BPV-1-specific probe. *Dpn*I cleaves its DNA recognition sequence, GATC, only when the adenine residue is methylated. DNA that has undergone replication in eukaryotic cells is no longer methylated at the adenine in this sequence context and is therefore resistant to *Dpn*I cleavage. As shown in Fig. 3A, replication of the parental plasmid, pMH142-6, containing the wild-type E1 gene, could be detected at 4, 5, and 6 days posttransfection. However, replication of the BPV-1 genomes carrying the E1 amino acid substitutions was not detected at any of the time points, indicating that the E1 mutants supported replication to a level less than 10% that of wild-type E1 (based upon PhosphorImager quantitation of the signal of wild-type BPV-1 replicon over background signal). In addition to these studies, we also looked at the replication competence of the E1 mutant genomes in stably transfected (focus-selected) mouse C127 cells. All E1 mutant genomes were defective (below detectable limits of one copy per cell) for plasmid replication (data not shown).

In the context of the viral genome, the E1 gene is thought to be expressed at low levels. To assess the replication competence of the mutant E1 proteins when more efficiently expressed, transient replication assays were performed in which the E1 and E2TA genes were expressed in *trans* from the cytomegalovirus (CMV) immediate-early promoter. The amino acid substitutions in E1 were moved into the plasmid pCGEag1235$^-$, which contains the E1 gene positioned downstream of the CMV immediate-early promoter. The 5′ splice signal within E1 at nt 1235 was mutated to inhibit expression of a truncated form of E1. This vector supports robust expression of E1 at levels greater than that achieved from the homologous BPV-1 promoters present in the intact BPV-1 genome. The plasmids pCGEag1235$^-$ and pCGE2 and the replicon pRLH89.7 were introduced into C33A cells by electroporation. The plasmid pCGE2 contains the E2TA gene positioned downstream of the CMV immediate-early promoter. A point mutation present in the E2TA gene in pCGE2, at BPV-1 nt 3092, replaces the initiation codon for the E2 repressor, E2TR, with one coding for isoleucine. The BPV-1 replicon pRLH89.7 contains BPV-1 sequences from nt 6958 to 93 (BPV-1 long control region [LCR]) and includes the minimal origin of replication (nt 7914

TABLE 1. Summary of properties of E1 mutants

Domain	E1 gene	DNA binding[a]	ATP binding[b]	In vivo protein[c]	Replication[d]		Fold E1 repression[e]
					Full length	In *trans*	
	Wild type	1.0	1.0	1.0	1.0	1.0	20 ± 5.6
I	W295R	<0.02	1.5 ± 0.3	0.3 ± 0.2	<0.1	<0.01	50 ± 1.7
II	N344H/A345E	0.7 ± 0.3	2.3 ± 1.3	0.4 ± 0.2	<0.16	<0.01	100 ± 33.3
None	R370T	0.5 ± 0.2	0.8 ± 0.3	1.0 ± 0.1	<0.1	0.1	142.8 ± 33.3
IV	L446K	0.4 ± 0.3	2.6 ± 1.1	<0.1	<0.1	<0.01	100 ± 33.3
V	F464C	0.5 ± 0.2	2.2 ± 1.9	1.0 ± 0.1	<0.1	<0.01	166.7 ± 50
V	L466H	0.2 ± 0.1	1.3 ± 0.16	<0.1	<0.1	0.3	1.7 ± 0.53
VI	D497A/G498A	1.4 ± 0.8	1.7 ± 1.1	0.1 ± 0.03	<0.1	0.1	4.2 ± 2.0
VII	N523I	1.0 ± 0.1	1.9 ± 1.6	<0.1	<0.16	<0.01	1.3 ± 1.0
VIII	F542V	0.7 ± 0.4	1.3 ± 0.7	0.1 ± 0.03	<0.1	0.3	0.3 ± 0.5

[a] DNA binding activity for wild-type E1 [(phosphorimager counts in origin-containing DNA band bound by wild-type E1) − (phosphorimager counts in nonspecific DNA band bound by wild-type E1)] was set at 1.0. The DNA binding activity of mutant E1 was determined as follows: [(phosphorimager counts in origin-containing DNA band bound by mutant E1) − (phosphorimager counts in nonspecific DNA band bound by mutant E1)]/[(phosphorimager counts in origin-containing DNA band bound by wild-type E1) − (phosphorimager counts in nonspecific DNA band bound by wild-type E1)]. Provided are the means and standard deviations for three independent experiments. A representative experiment is shown in Fig. 6.

[b] ATP binding by wild-type E1 (phosphorimager counts in ox-ATP wild-type E1 band) was set at 1.0. ATP binding for mutants was calculated as follows: (phosphorimager counts in full-length ox-ATP mutant E1 band)/(phosphorimager counts in ox-ATP wild-type E1 band). Signals were corrected for relative amounts of GST-fusion proteins present in each sample. Provided are the means and standard deviations from three independent experiments. A representative experiment is shown in Fig. 6.

[c] In vivo levels of E1 proteins represent the amount of mutant E1 protein present in transiently transfected COS cells detected by IP and/or Western blotting compared to that of wild-type E1 protein (data taken from multiple experiments). The data was quantitated by scanning the Western blot with a soft laser densitometer (Zenith).

[d] Replication activity of E1 proteins. The first column provides quantitation of data presented in Fig. 3A, day 6. The second column provides quantitation of data presented in Fig. 3B, high E1 dose. The replication activity of wild-type E1 [(phosphorimager counts in *Dpn*I-resistant DNA band) − (background)] was set at 1.0. The replication activity of mutant E1s was determined as follows: [(phosphorimager counts in *Dpn*I-resistant DNA band generated with mutant E1) − (background)]/[(phosphorimager counts in *Dpn*I-resistant DNA band generated with wild-type E1) − (background)].

[e] E1 inhibition of E2TA-mediated transcription from pRLH89.7 in BEF cells. Fold repression reflects [(luciferase activity of test plasmid) + E2 + (E1 TTL)]/[(Luciferase activity of test plasmid) + E2 + (indicated E1 gene)]. Provided are the means and standard deviations from three independent experiments. A representative experiment is shown in Fig. 5C.

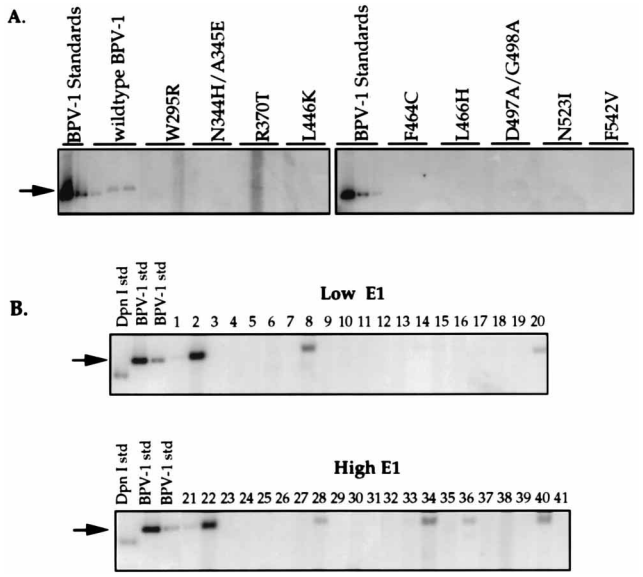


FIG. 3. Transient replication properties of E1 mutants. Shown are phosphorimages of BPV-1-specific Southern blots in which Hirt DNAs were resolved on 0.8% agarose gels. (A) Lanes marked BPV-1 Standards were loaded with 200, 20, and (in right image) 2 pg of pMH142-6 DNA linearized with *Hin*dIII. In each of the other lanes were run *Hin*dIII-*Dpn*I-digested Hirt DNAs from cells transfected with wild-type BPV-1 plasmid (pMH142-6) or derivatives of pMH142-6 that contain the indicated E1 mutations. Groups of three lanes represent samples from three individual transfections of each plasmid DNA extracted from cells at 4, 5, and 6 days posttransfection. The arrow indicates the migration of replicated DNA on the blot. (B) Lanes marked Dpn I std contain the plasmid pCGEag1235$^-$ digested with *Bgl*II and serve as a control for complete *Dpn*I digestion (absence of this *Bgl*II fragment in lanes 1 to 41 indicates that *Dpn*I digestion went to completion, because pCGEag1235$^-$ or its derivatives present in each transfection do not contain a BPV-1 origin). The lanes marked BPV-1 std contain 300 or 30 pg of pRLH89.7 linearized with *Bgl*II. The odd-numbered lanes contain *Dpn*I-*Bgl*II-digested Hirt DNAs from cells that were transfected with pRLH89.7 and 3 μg (Low E1, lanes 1 to 20) or 12 μg (High E1, lanes 21 to 40) of wild-type or mutant pCGEag1235$^-$. The even-numbered lanes contain *Dpn*I-*Bgl*II-digested Hirt DNAs from cells that were transfected with pRLH89.7, pCGE2, and 3 μg (Low E1) or 12 μg (High E1) of wild-type or mutant pCGEag1235$^-$. Samples run in lanes 1, 2, 21, and 22 were from cells that received the parental pCGEag1235$^-$ vector. All other numbered lanes received the following E1 mutant derivatives of pCGEag1235$^-$: lanes 3, 4, 23, and 24, W295R; lanes 5, 6, 25, and 26, N344H/A345E; lanes 7, 8, 27, and 28, R370T; lanes 9, 10, 29, and 30, L446K; lanes 11, 12, 31, and 32, F464C; lanes 13, 14, 33, and 34, L466H; lanes 15, 16, 35, and 36, D497A/G498A; lanes 17, 18, 37, and 38, N523I; and lanes 19, 20, 39, and 40, F542V. Lane 41 contains *Dpn*I-*Bgl*II-digested Hirt DNA from cells that were transfected with pRLH89.7 and pCGE2 only. The arrow indicates where replicated (i.e., *Dpn*I-resistant pRLH89.7) DNA migrated on the blots.

to 27). C33A cells were chosen for these experiments because it had been previously shown that C33A cells could support replication of a BPV-1 replicon when E1 and E2TA genes were expressed in *trans* (11). Three days posttransfection Hirt DNA was isolated, digested with *Bgl*II and *Dpn*I, and analyzed by Southern hybridization. The completion of *Dpn*I digestion was indicated for each sample by the absence of any undigested pCG vector DNA (the pCGEag1235$^-$ and pCGE2 vectors do not contain a BPV-1 origin of replication and are therefore sensitive to *Dpn*I digestion in C33A cells). Replication of the origin-containing plasmid, pRLH89.7, could be detected when 3 or 12 μg of wild-type E1 expression plasmid pCGEag1235$^-$ and 5 μg of E2 expression vector pCGE2 were introduced into C33A cells (Fig. 3B, lanes 2 and 22). *Dpn*I-resistant plasmid pRLH89.7 DNA was also detected when the E1 expression plasmid pCGEag1235$^-$ was cointroduced into C33A cells in the absence of the E2TA expression plasmid pCGE2 (Fig. 3B, lanes 1 and 21). Thus, a BPV-1 replicon can replicate in cells in the absence of E2TA when sufficient wild-type E1 protein is present. This result is consistent with that seen when the E1 gene of HPV-1 is overexpressed in similar transient replication assays in tissue culture cells (11) and correlates with the dispensability of E2TA in in vitro BPV-1 DNA replication assays (3, 27, 37, 47).

When efficiently expressed in *trans* from the CMV immediate-early promoter, several of the E1 mutants (R370T, F542V, L466H, and D497A/G498A) that did not support replication in the context of the full-length viral genome could support replication in C33A cells (Fig. 3B, lanes 8, 20, 28, 34, 36, and 40). This replication activity, however, was only partial compared to that of wild-type E1. DNA replication by three of the mutant E1 genes (R370T, F542V, and D497A/G498A) was only seen in the presence of E2, and the abundance of replicated DNA was less than that obtained with wild-type E1. These data indicate that these mutant E1 genes are only partially defective in replication activity. The partial defectiveness displayed by three of these mutant proteins (F542V, L466H, and D497A/G498A) may simply reflect the fact that they accumulate to lower levels in mammalian cells than does wild-type E1 (Fig. 2). No replication activity could be detected for the other five E1 mutant genes when expressed in *trans* in the presence or absence of E2TA (Fig. 3B). We performed similar studies in BEF cells; here none of the E1 mutants, when expressed in *trans*, could support replication of a BPV-1 replicon (data not shown). The fact that we did not see partial replication activity for the four mutants that supported replication at low levels in C33A cells likely reflects the lower transfection efficiency in BEF cells and the resulting lower signal-to-noise ratio for replicated DNA.

Dominant negative. It has been hypothesized that BPV-1 E1 can form multimers (22, 34). We hypothesized that if our replication-defective E1 point mutants could form multimers with wild-type E1, they might inhibit wild-type E1's ability to support replication. To address this possibility, the E1 mutant expression vectors were cotransfected with 3 μg of pCGEag1235$^-$, expressing wild-type E1, 5 μg of pCGE2, and 8 μg of the replicon pRLH89.7 at either an equal amount or two, four, or eight times the amount of wild-type pCGEag1235$^-$. Two of the five E1 mutants tested showed a dominant-negative phenotype (Fig. 4A). Coexpression of the E1 mutant N344H/A345E caused a reduction in viral DNA replication to less than 1% of that seen in its absence, while the mutant N523I led to a reduction to 10% of that seen in its absence (Fig. 4B). Other E1 point mutants (W295R, L446K, and F464C) did not have any detectable dominant-negative effect (data not shown). Wild-type E1 did not inhibit itself when wild-type pCGEag1235$^-$ was transfected at levels up to 15 μg (data not shown). The inhibition of replication seen with selected E1 mutants was not an artifact of introducing large amounts of plasmid DNA; total plasmid DNA content in all transfections was held constant (see Materials and Methods).

Repression of P_{89} promoter. BPV-1 E1 can specifically repress E2TA transactivation of the P_{89} promoter in BEF cells (30). To determine whether our mutant E1 proteins could repress E2TA transactivation, increasing amounts of pCGEag1235$^-$ plasmid, containing wild-type or mutant E1, were introduced into BEF cells by calcium phosphate transfection along with the plasmid pRLH89.7, containing the BPV-1 LCR with P_{89} promoter driving the luciferase reporter gene (Fig. 5A), and pC59, an E2TA expression plasmid. E2TA transactivated the P_{89} promoter 100-fold, and wild-type E1 was able to repress E2TA's activity to 6% of that seen in its absence (Fig. 5B). The E1 mutants W295R, N344H/A345E, R370T,

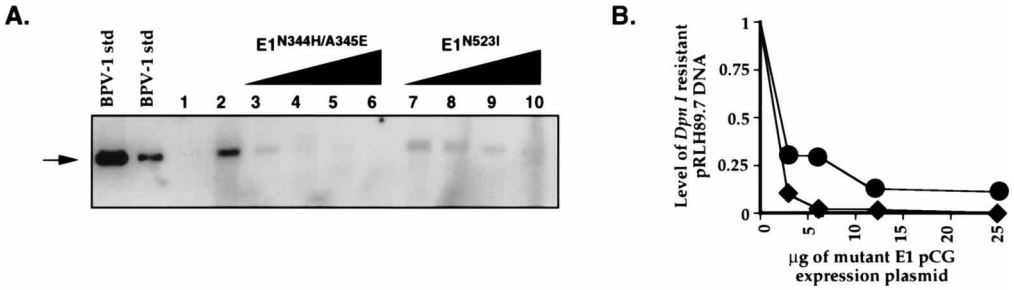


FIG. 4. Dominant-negative activity of specific BPV-1 E1 mutants. (A) Autoradiograph of a BPV-1-specific Southern blot in which Hirt DNAs were resolved on an 0.8% agarose gel. The lanes marked BPV-1 std contain 300 or 30 pg of pRLH89.7 linearized with *Bgl*II. Lanes 1 to 10 contain *Dpn*I-*Bgl*II-digested Hirt DNA from cells that were transfected with pRLH89.7 and pCGEag1235$^-$ (lane 1); pRLH89.7, pCGEag1235$^-$, and pCGE2 (lane 2); pRLH89.7, pCGEag1235$^-$, pCGE2, and increasing amounts (3, 6, 12, or 24 μg, respectively) of the plasmid pCGEag1235$^{-\ N344H/A345E}$ (lanes 3 to 6); and pRLH89.7, pCGEag1235$^-$, pCGE2, and increasing amounts (3, 6, 12, or 24 μg, respectively) of the plasmid pCGEag1235$^{-\ N523I}$ (lanes 7 to 10). The arrow indicates where replicated (i.e., *Dpn*I-resistant pRLH89.7) DNA ran on the blot. (B) Quantitation of relative levels of replicated pRLH89.7 DNA as a function of the amount (in micrograms) of pCGEag1235$^{-\ N344H/A345E}$ (◆) or pCGEag1235$^{-\ N523I}$ (●) plasmid DNA cotransfected into cells. (Quantitation was performed on the Southern blot shown in panel A. The level of replicated DNA in lane 2 was set at 1.0.)

L446K, and F464C could repress E2TA transactivation of the P_{89} promoter as well as wild-type E1 (Fig. 5C) and displayed a dose-response curve similar to that seen with wild-type E1 (data not shown). The E1 point mutants L466H, D497A/G498A, N523I, and F542V failed to repress E2TA transactivation of the P_{89} promoter (Fig. 5C). They all cluster in the C-terminal third of E1.

DNA binding. BPV-1 E1 has been shown to bind to the origin of viral DNA replication (BPV-1 nt 7781 to 83) (18, 41, 45). This binding is important for E1's role in viral DNA replication (18). To determine if our E1 mutants could bind the origin, a modified McKay assay (25) was performed. GST-E1 fusion proteins (containing wild-type or mutant E1) were synthesized in bacteria, bound to glutathione beads, and washed. BPV-1 DNA that had been digested with *Ava*II and α^{32}P end labeled was added to each GST-E1 preparation and incubated at room temperature. DNAs that were bound to the GST-E1 after extensive washing were extracted and analyzed on a polyacrylamide gel. The 219-bp BPV-1 DNA fragment containing the origin bound to the wild-type GST-E1 at levels 50-fold over that measured with the control GST fusion protein, GST–β-globin (data not shown). The amount of the origin-containing fragment bound was quantitated by phosphorimage analysis and corrected for the amount of GST protein present in the Coomassie-stained gel (see Materials and Methods). The E1 mutant W295R did not bind to the origin-containing fragment with high specificity; its binding activity was reduced to 2% of that of wild-type E1 protein (Fig. 6A and Table 1). This result is consistent with the DNA binding domain in BPV-1 E1 mapping to amino acids 1 to 299 (18, 31, 41). Nonetheless, W295R was able to repress E2TA transactivation as well as wild-type E1 (Fig. 5C and Table 1). Thus, efficient DNA binding by E1 alone is not required for it to inhibit E2TA-mediated transcription. This finding is consistent with that of Le Moal et al. (17). Of the other mutant E1 proteins, all bound DNA with efficiencies close to that of wild-type E1 protein (i.e., within two standard deviations of wild-type E1 values) with the exception of one, L466H, which bound at levels of 20% of that of wild-type E1 (Fig. 6A and Table 1).

ATP binding. Both BPV-1 E1 and SV40 T-ag bind ATP (40) and have ATPase activity (23, 47). These biochemical properties are important for BPV-1 E1's and SV40 T-ag's roles in DNA replication. The amino acid substitutions generated in E1 are in regions of similarity with SV40 T-ag which have been shown to be involved in ATP-binding and/or ATPase activity in T-ag. To determine if our amino acid substitutions altered E1's ability to bind ATP, we performed affinity labeling experiments with a radiolabeled, oxidized derivative of ATP (ox-ATP) which can be coupled covalently to primary amines on ATP binding proteins. ATP binding reactions were performed with GST-E1 fusion proteins (mutant or wild-type E1) that were bound to glutathione beads and then analyzed by SDS-PAGE (Fig. 6B). The amount of ATP bound was quantified by phosphorimage analysis and corrected for the amount of GST-E1 fusion protein present (see Materials and Methods). Wild-type E1 bound ox-ATP 20-fold over the amount bound by GST–β-globin (Fig. 6B). The E1 mutants were able to bind ox-ATP at levels that were from 80 to 260% of that of wild-type GST-E1 (Table 1). Therefore, none of our E1 mutants appear to be disrupted grossly for binding ATP.

DISCUSSION

Clertant and Seif (6) first reported the amino acid similarity between BPV-1 E1 and SV40 T-ag. The similarity between the two proteins occurs in the region that in SV40 T-ag has been shown to contain the domains necessary for ATP binding, ATPase-dependent DNA helicase activity, and DNA polymerase alpha binding, all biochemical properties important for SV40 T-ag's role in viral DNA replication. Therefore, we predicted that amino acid substitutions introduced into the E1 gene product at residues of identity with T-ag would disrupt E1's ability to replicate the genome. This result was observed when mutant E1 genes were expressed in the context of the full-length viral genome (see the quantitative summary of data for each mutant in Table 1). Five of the nine mutants (W295R, N344H/A345E, L446K, F464C, and N532I) also displayed a replication-defective phenotype when efficiently expressed in *trans* in C33A cells, while four displayed partial activity. These results demonstrate that the amino acids conserved between E1 and SV40 T-ag are important for E1's ability to replicate the viral genome. Interestingly two of the E1 mutants that were completely defective for supporting viral DNA replication could act in *trans* to inhibit replication by wild-type E1. E1 also can inhibit E2TA-mediated transcription. We found that five E1 mutants that were defective or greatly reduced in their ability to support viral DNA replication were competent to inhibit E2TA-mediated transcription. Thus, properties of E1 important in viral DNA replication are not necessary for its inhibition of E2TA-mediated transcription; of these, one is DNA binding.

Six of the nine E1 mutant proteins (W295R, N344H/A345E,

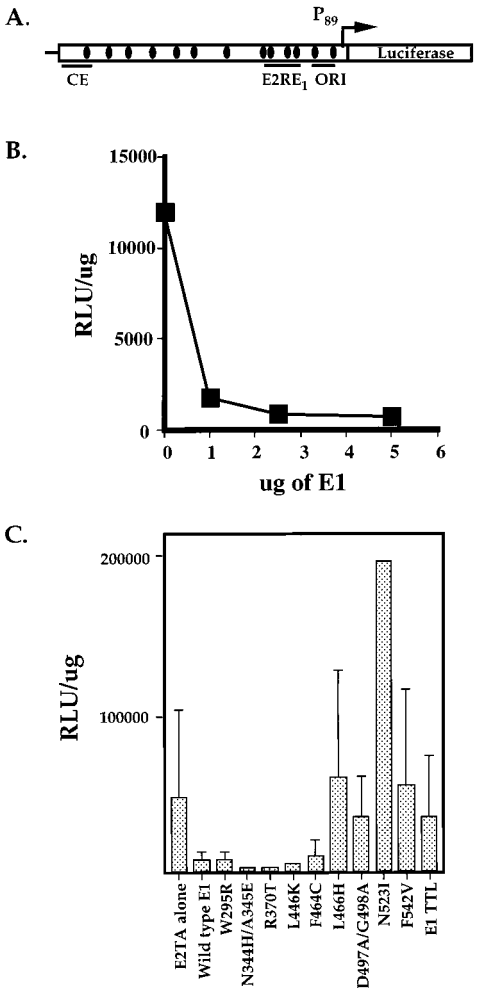


FIG. 5. Inhibition of E2TA-mediated transcription. (A) Structure of reporter plasmid pRLH89.7. The left box represents BPV-1 LCR sequences (nt 6958 to 93). The black ovals represent the positions of the E2 binding sites in the LCR (19). Positions of the four E2 binding sites which comprise the BPV-1 E2-dependent enhancer element, $E2RE_1$ (38), of the bovine constitutive enhancer, CE (44), and the replication origin, ORI (43), are indicated. The BPV-1 P_{89} promoter is indicated by the arrow. (B) Repression of E2-transactivated pRLH89.7 by wild-type BPV-1 E1 in BEF cells. Each transfection received 5 μg of pRLH89.7 and 0.2 μg of the E2-expressing plasmid pC59. The amount of pCGEag1235$^-$ included in different transfections is indicated on the *x* axis. Luciferase assays (Promega) were performed according to the manufacturer's instructions. Luciferase activity (*y* axis) is given in relative light units (RLU) per microgram of protein in the lysate. Luciferase activity in cells transfected with pRLH89.7 in the absence of pC59 was approximately 150 RLU/μg (data not shown). (C) Repression of E2-dependent luciferase expression from pRLH89.7 by wild-type or mutant BPV-1 E1. Shown are the average luciferase activities and range of values for transfections performed in triplicate within a single experiment. Each transfection received 5 μg of pRLH89.7, 0.2 μg of the E2-expressing plasmid pC59, and 5 μg of E1 expression plasmid. Not shown is the range of activity values for N523I (±130,000). Quantitation of the inhibition of E2TA-mediated transcription data from multiple experiments is provided in Table 1 (fold E1 repression). Error bars indicate standard deviations.

R370T, N523I, L446K, and F464C) displayed robust activities when expressed in mammalian cells (i.e., efficient inhibition of E2TA-mediated transcription [W295R, N344H/A345E, R370T, L446K, and F464C] or dominant-negative activity in replication [N344H/A345E and N523I]). The remaining three mutants (L466H, D497A/G498A, and F542V) displayed reduced activity or no detectable activity. These three mutants also were among those that accumulated to low levels in COS cells (Fig. 2 and Table 1), raising the possibility that differences in steady-state levels of E1 protein may account for their defective phenotypes. Low protein accumulation likely accounts for the phenotypes of at least one (D497A/G498A) of these three mutants, since the level of expression of this protein correlated with its reduced activity in replication and because no biochemical defects (e.g., ATP binding and DNA binding) were noted (Table 1). However, the other two mutants (L466H and F542V) likely are phenotypically defective for additional rea-

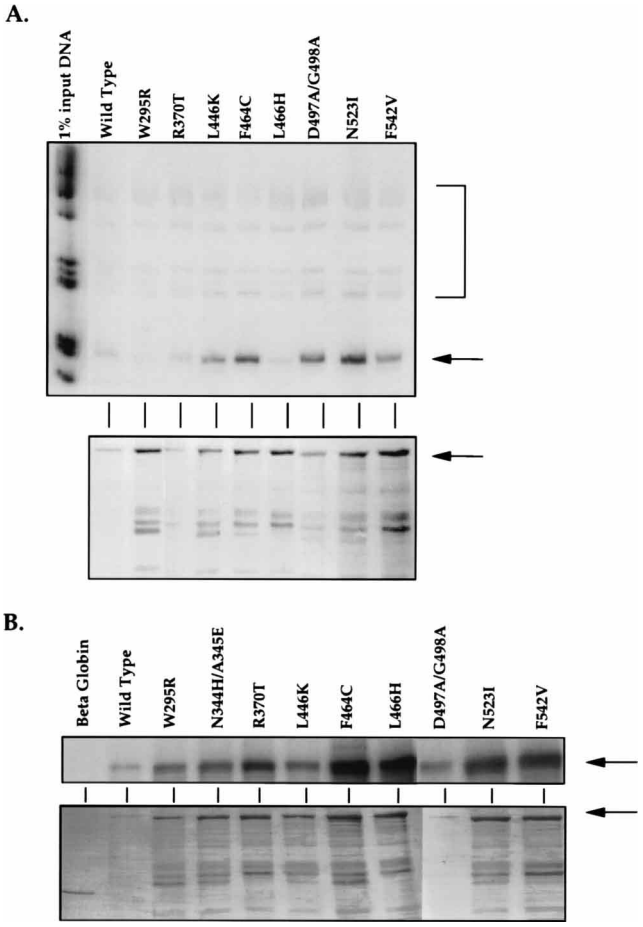


FIG. 6. DNA binding and ATP binding activities of E1 mutants. (A) DNA binding by E1 mutants. Shown is an autoradiograph (top) of a representative McKay assay. In this experiment 250 ng of GST-E1 protein was incubated with BPV-1 DNA that had been digested with *Ava*II and end labeled with [α-^{32}P] dCTP. The arrow indicates the origin-containing fragment. The bracket indicates DNA bands that do not contain the origin. Size markers for DNA fragments are on the left. In the Coomassie-stained gel (bottom) of GST-E1 fusion proteins that were prepared in parallel with the GST-E1 fusion proteins used in the DNA binding experiment, the arrow indicates migration of full-length GST-E1 fusion protein. The DNA binding efficiency of each fusion protein was determined by measuring the level of origin signal to nonspecific signal bound by phosphorimage analysis (top) and correcting it for the amount of fusion protein detected by Coomassie staining (bottom). Quantitation of DNA binding data from multiple experiments is provided in Table 1 (DNA binding). (B) ATP binding by E1 mutants. (Top) Autoradiograph from a representative ATP binding assay. In this experiment 250 ng of GST-E1 protein was incubated with ox-[^{32}P]ATP. The arrow indicates full-length GST-E1 protein bound to ox-[^{32}P]ATP. (Bottom) Coomassie blue-stained image of the same gel prior to drying. The arrow indicates migration of the full-length GST-E1 fusion proteins. The ATP binding efficiency of each fusion protein was determined by measuring the level of the E1-bound ox-[^{32}P]ATP signal by phosphorimage analysis (top) and correcting it for the amount of fusion protein detected by the Coomassie blue staining (bottom) of the SDS-PAGE gel. Quantitation of ATP binding data from multiple experiments is provided in Table 1 (ATP binding).

sons. L466H, which could partially support viral DNA replication when efficiently expressed in *trans* (Fig. 3 and Table 1), is reduced in its DNA binding activity to 20% that of wild-type E1 protein (Table 1). In that assay, levels of DNA binding were corrected for levels of E1 protein in the assay. Since E1's DNA binding activity appears to contribute to DNA replication (e.g., see the correlation between defectiveness in DNA binding and replication for W295R in Table 1), this defect likely contributes to the partial replication defect. The other mutant E1 protein, F542V, which accumulated to a level of 10% that of wild-type E1 in COS cells and could support replication in *trans* to a level of 30% that of wild-type E1, nevertheless was completely defective in inhibiting E2TA-mediated transcription (Fig. 5C and Table 1). We should have been able to detect any partial inhibition of E2TA-mediated transcription; therefore, we conclude that this mutant is defective in the latter activity. All of the mutant proteins that accumulated to low levels in COS cells had amino acid substitutions in the C-terminal one-third of E1. Three of the four most-C-terminal mutants (D497A/G498A, N523I, and F542V), all of which displayed lowered levels of protein accumulation compared to that of E1, also were temperature sensitive for their replication phenotype (data not shown). Other mutants failed to display a temperature-sensitive phenotype. One possible interpretation of these findings is that the C-terminal amino acid substitutions induce changes in E1's protein folding that lead to protein instability and loss of function at 37 but not at 32°C.

We had predicted that replication-defective mutants might be able to inhibit wild-type E1 by forming mixed multimers. Two of our E1 mutants (N344H/A345E and N523I) displayed such a dominant-negative phenotype (Fig. 4). By contrast other replication-defective mutants (W295R, L446K, and F464C) did not display a dominant-negative activity in replication assays even though their expression was detected in mammalian cells (Fig. 2 and Table 1) and they possessed other activities, i.e., inhibition of E2TA-dependent transcription (Fig. 5 and Table 1). The absence of dominant-negative activity by these mutant proteins may be indicative of a defect in multimerization. For example, W295R binds to the origin-containing DNA fragment very inefficiently (Fig. 6A and Table 1). It has been proposed that E1 must bind to the BPV-1 origin in order to form a multimeric complex (22, 35). Thus, W295R would be predicted not to form multimers. The other two mutants (L446K and F464C) were able to bind DNA in a McKay assay (Fig. 6A and Table 1). We speculate that these closely spaced mutants define a region in E1 that contributes to multimerization.

Our results demonstrate that at least a subset of the properties of E1 required for supporting viral DNA replication are not required for modulating viral transcription. Five E1 mutants (W295R, N344H/A345E, R370T, L446K, and F464C) could inhibit E2TA-mediated transcription. Of these mutants, four possessed no detectable replication activity and the other one had greatly reduced replication activity in C33A and BEF cells. Thus, E1's ability to inhibit E2TA-mediated transactivation of P_{89} is not dependent on E1's ability to replicate the viral genome. This conclusion is consistent with those of Sandler et al., who demonstrated that E1 could inhibit E2TA transactivation even with an E2TA mutant that could not support viral DNA replication (30). Based upon our study we predict that DNA binding (demonstrated to be deficient in W295R [Fig. 6 and Table 1]) and E1's homomultimerization (predicted to be defective in L446K and F464C [see above]) are E1 properties required for DNA replication but not for inhibition of E2TA transactivation. Transactivation by E2TA is thought to be mediated, at least in part, by its physical association with cellular transcription factors (29, 39). Thus, the ability of E1 to bind E2TA and/or displace E2TA-associated cellular transcription factors may contribute to its inhibition of E2TA-mediated transcription. In this regard it is interesting that all four E1 mutants that were defective for the inhibition of E2TA-mediated transcription (L466H, D497A/G498A, N523I, and F542V) lie within the C-terminal one-third of E1, well within the domain of E1 shown by Sarafi and McBride (31) and Lusky and Fontane (21) to be required for the cooperative binding of E1 and E2 to the origin.

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